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A review of sustainable hydrogen production using seed sludge via dark fermentation

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ABSTRACT

In recent years, the production of hydrogen (H_2) via dark fermentation has become increasingly popular because it is a sustainable approach to produce clean energy. This review presents an overview with a critical analysis of the technical challenges in obtaining high H_2 yield through dark fermentation. Particular focus is given to the pretreatment methods that affect H_2 production. We observed that heat pretreatment is the most frequently applied and the most effective method of eliminating H_2-consuming bacteria (HCB) while preserving H_2-producing bacteria (HPB). The pre-dominant HPB species after pretreatment belongs to the genus *Clostridium* and hence the fermentation conditions are optimized according to their preference for H_2 production. Besides, we also reviewed fermentation conditions such as substrate, pH, temperature, oxidation–reduction potential (ORP), types of nutrient and inhibitor substrate, to obtain clearer insight on the influences of critical parameters in H_2 production.

Contents

Abbreviations: H_2, hydrogen; HPB, hydrogen producing bacteria; HCB, hydrogen consuming bacteria; POME, palm oil mill effluent; UASB, up flow anaerobic sludge blanket; TVS, total volatile solid; VS, volatile solid; BES, 2-bromoethanesulfonate; MSW, municipal solid waste; VFA, volatile fatty acid

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1. Introduction

Hydrogen gas (H_2) is an alternative fuel to reduce the over reliance on fossil fuels as the primary energy used in vehicles and machines. Generally, H_2 fuel can be used in conventional gasoline engines with modifications in order to generate energy via combustion in the air [1,2]. The combustion of H_2 is sustainable and environmentally friendly because it does not generate greenhouse gases such as carbon dioxide and methane [3,4]. Hydrogen also possesses high energy yield (141.9 J/kg) among the known fuel types such as methane (55.7 J/kg), natural gas (50 J/kg), biodiesel (37 J/kg) and ethanol (29.9 J/kg) [5]. However, more than 96% of global H_2 is generated from fossil fuels [6,7]. Therefore, there is an urgency to develop a more cost-effective and environmentally friendly technology to for H_2 production.

Dark fermentation is a biological approach commonly used to produce H_2 in the absence of light [8]. This process does not require solar input and hence the configuration of the bioreactor is simpler and cheaper [9]. Most importantly, this technology has attracted attention because it can use a versatile range of substrate, particularly renewable resources that are organically rich such as stillage, sludge, leachate, pomace, stalks and bagasse [10–12]. Due to cost and environmental concerns, organic waste material is a better choice of substrates than pure compounds such as sugar or starch. This technology allows dark fermentation to be integrated into wastewater treatment systems to produce H_2 and to treat wastewater.

Seed sludge contains diverse microflora that can produce H_2 via dark fermentation [13–16]. Microorganisms found in the seed sludge are more beneficial than pure cultures because they are more adaptive to environmental stresses including limited substrates, and changes in pH and temperature. Moreover, the diverse microflora present in the seed sludge might provide synergistic interactions that improve substrate degradation and thus enhance H_2 production. Unfortunately, microflora in the seed sludge usually consists of both H_2-consuming and H_2-producing bacteria (Table 1). Therefore, it is essential to eliminate the activity of H_2-consuming bacteria (HCB) in order to increase H_2 production from H_2-producing bacteria (HPB). To achieve this, seed sludge can be pretreated using various physical and chemical pretreatment methods to enrich HPB. However, the search for the most effective pretreatment method for this purpose is still under intensive research.

Apart from the variety of HPB involved in dark fermentation, high H_2 yield is also associated with fermentation conditions including pH, temperature and types of substrate. These factors influence H_2 production by altering the physiological properties such as the enzymatic activities of HPB. In addition, H_2 production can be further enhanced by supplements or constrained by inhibitors. Theoretically, a maximum of 12 mol of H_2 is produced from 1 mol of glucose.

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2 \quad (1)$$

However, currently the highest reported H_2 yield is only about 20% of this maximum yield. Therefore, in order to improve H_2 yield, it is important to recognize the major contributing factors in H_2 production.

This paper critically reviews the challenges of H_2 production using seed sludge as inoculum, focusing mainly on (1) the strengths and weaknesses of different pretreatment methods on the seed sludge; and (2) the effects of different factors including types of potential substrate, operation conditions, nutrients and inhibitors, and the diverse microflora in seed sludge.

2. Factors affecting hydrogen production by seed sludge

2.1. Effects of sludge pretreatment

In order to enhance H_2 production, pretreatment is commonly used to enrich HPB. Pretreatment must be able to selectively preserve HPB while eliminating HCB. Untreated seed sludge generally produces low H_2 yield (<1.0 mol H_2/mol glucose) and pretreated seed sludge successfully improves H_2 yield (Supplementary Tables S1–S4). This is verified by the hydrogenase

Table 1
Example of H_2 producing and consuming bacteria with their characteristics.

Organisms	Functions	Characteristics	Ref.
Clostridium spp.	H_2 production	Obligate and mesophilic anaerobes The most popular H_2 producer Ferment a wide range of carbohydrates and produce H_2 E.g. *Clostridium butyricum, C. acetobutylicum, C. tyrobutyricum, C. saccharolyticum*	[17–20]
Thermoanaerobacterium spp.	H_2 production	Obligate and thermophilic anaerobes E.g. *Thermoanaerobacterium thermosaccharolyticum*	[21]
Ethanoligenens spp.	H_2 production	Obligate anaerobes Produce solvent during H_2 production E.g. *Ethanoligenens harbinens*	[22]
Bacillus spp.	H_2 production	Facultative anaerobes May possess important features such as salt tolerance E.g. *Bacillus megaterium*	[23]
Enterobacter spp.	H_2 production	Facultative anaerobes Have better tolerance against oxidative stress E.g. *Enterobacter aerogenes*	[22]
Klebsiella spp.	H_2 production	Facultative anaerobes Have better tolerance against oxidative stress E.g. *Klebsiella pneumonia*	[24]
Methanogens	H_2 consumption	Obligate anaerobes Utilize H_2 for methane production E.g. *Methanobacterium* spp., *Methanococcus* spp. etc.	[25]
Other H_2 consuming bacteria	H_2 consumption	Obligate/facultative anaerobes Utilize H_2 as electron donor and precursors for metabolic compounds E.g. *Lactobacillus* spp. and *Bifidobacterium* spp.	[26,27]

Table 2
Comparison of H_2 yield between heat pretreated and untreated sludge.

H_2 yield		Pretreatment condition	Sludge source	Ref.
Heat pretreatment	Untreated			
1. 2.38 mol H_2/mol glucose	N.A.	100 °C, 15 min	Sewage treatment plant	[29]
2. 2.30 mol H_2/mol glucose	0.43 mol H_2/mol glucose	65 °C, 30 min	Sewage treatment plant	[18]
3. 1.95 mol H_2/mol glucose	0.43 mol H_2/mol glucose	95 °C, 30 min	Sewage treatment plant	[18]
4. 1.04 mol H_2/mol glucose	0.70 mol H_2/mol glucose	70 °C, 30 min	Sewage treatment plant	[30]
5. 0.90 mol H_2/mol glucose	0.38 mol H_2/mol glucose	95 °C, 30 min	Sewage treatment plant	[31]
6. 0.40 mol H_2/mol glucose[b]	0.20 mol H_2/mol glucose[b]	100 °C, 30 min	Intertidal zone	[27]
7. 1.61 mol H_2/mol hexose	0.3 mol H_2/mol hexose	100 °C, 60 min	POME treatment plant	[21]
8. 0.0106 mol H_2/ g carb.[a]	0.0191 mol H_2/g carb.[a]	70 °C, 30 min	Sewage treatment plant	[14]
9. 0.0000122 mol H_2/g COD	0.0018 mmol H_2/g COD	100 °C, 60 min	H_2 producing reactor	[32]
10. 0.00041 mol H_2/g COD	0.00012 mol H_2/g COD	100 °C, 60 min	POME treatment plant	[33]
11. 0.00233 mol H_2/g VS[a]	0.0265 mol H_2/g VS[a]	90 °C, 60 min	Anaerobic treatment plant	[34]
12. 0.0498 mol H_2[a]	0.0341 mol H_2[a]	100 °C, 90 min	Intertidal zone	[35]
13. 0.0488 mol H_2[a]	0.0366 mmol H_2[a]	80 °C, 20 min	Intertidal zone	[23]
14. 0.0325 mol H_2[a]	0.0366 mmol H_2[a]	100 °C, 20 min	Intertidal zone	[23]

[a] H_2 yield was estimated using $PV=nRT$ at standard condition where $P=1$ atm; $R=8.21\times10^{-5}$ m^3 atm/mol K and $T=300$ K.
[b] Estimated value.

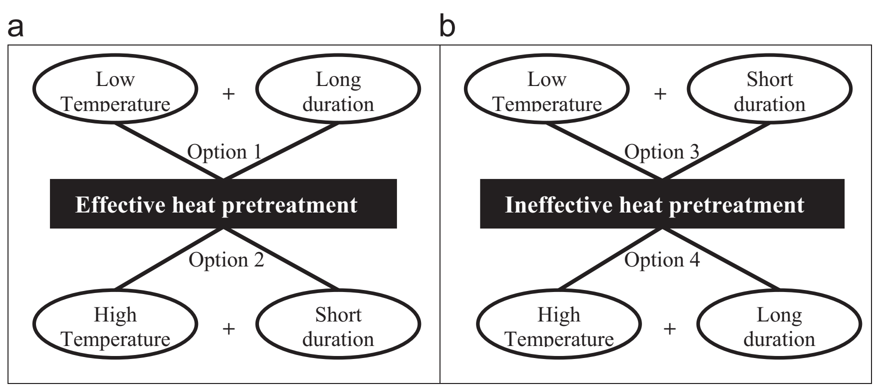


Fig. 1. Relationship between pretreatment temperature and duration. (a) Effective heat pretreatment is resulted from the combination of low temperature with long duration (option 1) or high temperature with short duration (option 2); (b) ineffective heat pretreatment is resulted from the combination of low temperature with short duration (option 3) or high temperature with long duration (option 4).

(primary H_2-producing enzyme) activity in the pretreated seed sludge that has been reported to be three fold higher when compared to the untreated seed sludge [28]. These indicate that the pretreatment successfully enriches HPB and increased H_2 yield.

The pretreatment methods are divided into physical and chemical pretreatments. Physical pretreatments are further separated into heat, ultrasonication, ultraviolet irradiation, aeration, and freeze and thaw methods, while chemical pretreatments include pH pretreatment, and chemical activation and inhibition. The selection of pretreatment methods is important because bacteria react differently to the stress applied. For example, it has been shown that acid pretreated seed sludge that was dominated by HCB, such as *Propionibacterium granulosum*, produced 10.4 fold less H_2 compared to heat treated seed sludge [22]. This suggests that heat pretreatment is the most effective method to eliminate *P. granulosum*. It demonstrates that the type of pretreatment serves an important role in controlling H_2 yield as it directly affects the variety of bacteria that is present in the seed sludge.

2.1.1. Physical pretreatments

2.1.1.1. Heat pretreatment. In physical pretreatment, heat pretreatment is the most commonly used method (Supplementary Table S2). This is a simple method that eliminates HCB effectively and has a high potential for commercialization. A review of studies shows that the highest H_2 yield was produced by seed sludge pretreated at 65 °C which yielded 2.30 mol H_2/mol, that is 8.85 fold higher than the untreated sludge (Table 2, no. 2). Seed sludge pretreated at this temperature preserved the most types of HPB, and no HCB were detected [18]. This indicates that heat pretreatment successfully eliminated HCB and improved H_2 production. On the other hand, heat pretreated seed sludge also significantly enhances the reduction of chemical oxygen demand (COD) in wastewater. It has been shown that COD of POME was reduced up to 89% and H_2 yield was increased 3.4 fold compared to that of untreated seed sludge [33]. The reduction of COD in wastewater during H_2 production signifies the potential of applying heat pretreated sludge for wastewater treatment via dark fermentation.

It is challenging to identify the best combination of pretreatment temperature and heating duration in order to further improve H_2 yields (Fig. 1). Among the reported combinations, seed sludge pretreated at 65 °C for 30 min and 100 °C for 15 min are the most promising combinations (Table 2, nos. 1 and 2). This suggests that a lower pretreatment temperature may require a longer pretreatment time and vice versa in order to achieve similar H_2 yields. In contrast, preheating seed sludge at higher temperatures for longer durations has shown lethal effects on HPB which reduces H_2 yield [18,23,36]. For example, in one study seed sludge pretreated at 70 °C for 30 min produced even lower H_2 than the untreated seed sludge (Table 2, no. 8). Similarly, H_2 produced by seed sludge pretreated at 95 °C was 4.53 fold lower than seed sludge pretreated at 65 °C (Table 2, nos. 2 and 5). This variation between H_2 productions and pretreatment combinations could be

Table 3
H_2 production from sludge pretreated with ultrasonication, ultraviolet irradiation, aeration and freeze and thaw method.

Pretreatment condition	H_2 yield	Sludge source	Ref.
Ultrasonication			
1. Ultrasonic, 20 min	1.03 mol H_2/mol glucose	Sewage treatment plant	[30]
2. Ultrasonic, 30 min	0.00016 mol H_2/g VS[a]	Sewage treatment plant	[45]
3. Ultrasonic, 30 min	0.00423 mol H_2/g TS[a]	Sewage treatment plant	[41]
4. Ultrasonic, 30 min	0.00126 mol H_2/g COD[a]	Sewage treatment plant	[46]
5. Ultrasonic, 30 min	0.0058 mol H_2/g cornstalk[a]	Sewage treatment plant	[12]
Ultraviolet irradiation			
6. UV, 15 min	0.00565 mol H_2/g TS[a]	Sewage treatment plant	[41]
7. UV, 15 min	0.00434 mol H_2/g TS[a]	Sewage treatment plant	[41]
Aeration			
8. Aeration, 24 h	0.70 mol H_2/mol glucose	Sewage treatment plant	[31]
9. Aeration, 24 h	0.00406 mol H_2/ g glucose[a]	Sewage treatment plant	[47]
10. Aeration, 12 h	0.00912 mol H_2[a]	Sewage treatment plant	[22]
Freeze and thaw			
11. Freeze (−25 °C, 24 h) and thaw (R.T., 5 h)	~0.15 mol H_2/mol glucose[b]	Intertidal zone	[27]
12. Freeze (−10 °C, 24 h) and thaw (30 °C)	0.00019 mol H_2/g COD	POME treatment plant	[33]

[a] H_2 yield was estimated using $PV=nRT$ at standard condition where $P=1$ atm; $R=8.21\times10^{-5}$ m^3 atm/mol K and $T=300$ K.
[b] Estimated value.

due to the density of cells and the type of microorganisms present in the seed sludge [37,38]. It is reasonable to assume that seed sludge that contains higher cell density requires longer pretreatment durations to ensure all HCB are eliminated. However, the appropriate pretreatment combination is complicated due to the variety of bacteria present in the seed sludge. Depending on the source of the seed sludge, HCB such as Homoacetogens can survive under intensive heat, while HPB such as *Enterobacter* spp. is easily destroyed during heat pretreatment [31,39]. Therefore, the optimum heat pretreatment temperature and time are dependent on the types of HPB and HCB present in the seed sludge.

2.1.1.2. Ultrasonication. Ultrasonication uses sound waves to eliminate HCB. A summary of the H_2 yield using this pretreatment is listed in Table 3. This method eliminates HCB by destroying their cell walls. Although the same damage may also occur to HPB, this can be prevented by controlling the pretreatment duration and intensity [40,41]. Ultrasonic pretreatment improves H_2 production because ultrasonic waves break the sludge particles into smaller sizes, disintegrate coenobium and increase the interaction between the HPB and the substrate [42,43]. Studies have shown the maximum yield obtained from this method was only 1.03 mol H_2/mol glucose. This yield was only about 8% of the maximum H_2 yield that can be produced from 1 mol of glucose (Eq. (1)). This shows that this method is less satisfactory than heat pretreatments. Therefore, optimization studies are required, including studies on the power of the ultrasonic waves, time of exposure, and heat control, before this pretreatment method can produce satisfactory results.

2.1.1.3. Ultraviolet irradiation. Ultraviolet irradiation has bactericidal action that can eliminate HCB and enrich HPB. The recommended pretreatment condition is 15 min of UV irradiation. This condition has successfully eliminated methanogens and increased H_2 yield 0.39 fold compared to untreated sludge (Table 3, no. 7). However, the radiation can only be transmitted through the smaller sludge particles that are lighter in color. Hence, HCB inside the larger and darker colored sludge particles are protected from UV irradiation and only HCB present on the surface are eliminated [44]. This method is not as efficient as other physical treatments.

2.1.1.4. Aeration. Aeration pretreatment applies oxidative stress to deactivate anaerobic HCB. This pretreatment method is aimed at eliminating methanogens that are sensitive to oxidative stress. However, oxidative stress also damages obligate anaerobic HPB and is less effective against facultative HCB. The inefficiency of aeration pretreatment has been shown to result in low H_2 yield (0.7 mol H_2/mol glucose) that is far below the maximum H_2 yield (Table 3, no. 8). Hence, this may not be an effective method to enrich HPB in most of the seed sludge.

2.1.1.5. Freeze and thaw. This pretreatment method involves freezing and thawing seed sludge simultaneously at an extreme temperature. The freeze and thaw method appears to be the least effective physical pretreatment because seed sludge pretreated using this method has been shown to produce very low H_2 yield (0.15 mol H_2/mol glucose) compared to other pretreatment methods (Table 3, nos. 11 and 12). Freezing and thawing seed sludge instantaneously lysed the bacteria, including both HCB and HPB, which in turn reduced H_2 yield. Therefore, the freeze and thaw method is not a favored method to enrich HPB due to its detrimental effects on HPB cells.

2.1.2. Chemical pretreatments

2.1.2.1. pH pretreatment. The most popular chemical pretreatment method used to enrich HPB is pH pretreatment. This method involves adjusting the pH of seed sludge to an extreme pH such as pH 3 or 12 (Table 4) and attempting to lyse HCB. In principle, extreme pH induces HPB to form spores. Bacterial spores have rigid cell walls that are difficult to break and hence HPB should survive this pretreatment. However, in reality, most HCB do not form spores and their cell walls are easily disrupted at extreme pH levels [27,30,31,48]. Evidently, an acidic pretreatment is more effective compared to an alkali pretreatment. This is evidenced by the H_2 yield produced by acidic pretreated seed sludge which was found to be 1.67 fold higher than alkaline pretreated seed sludge (Table 4 nos. 2 and 19). This suggests that HPB is more susceptible to alkaline pretreatment but the reason is unknown as there are no reports on the effects of alkalinity on HPB survival. Nonetheless, pH pretreatment is still not as effective as heat pretreatment because the H_2 yield is not as high.

Table 4
Comparison of H_2 yield between pH pretreatment and untreated sludge.

H_2 yield		Pretreatment condition	Sludge source	Ref.
pH pretreatment	Untreated			
Acid pretreatment				
1. 2.25 mol H_2/mol glucose	N.A.	Acid (pH 3), 24 h	Sewage treatment plant	[48]
2. 1.51 mol H_2/mol glucose	0.38 mol H_2/mol glucose	Acid (pH 3), 24 h	Sewage treatment plant	[31]
3. 1.11 mol H_2/mol glucose	0.70 mol H_2/mol glucose	Acid (pH 3), 24 h	Sewage treatment plant	[30]
4. 0.85 mol H_2/mol glucose[b]	0.20 mol H_2/mol glucose[b]	Acid (pH 3–4), 24 h	Intertidal zone	[27]
5. 0.65 mol H_2/mol hexose	0.3 mol H_2/mol hexose	Acid (pH 3–4), 24 h	POME treatment plant	[21]
6. 0.0018 mol H_2/gCOD	0.0018 mmol H_2/g COD	Acid (pH 5), 24 h	H_2 producing reactor	[32]
7. 0.00032 mol H_2/g COD	0.00012 mol H_2/g COD	Acid (pH 3), 24 h	POME treatment plant	[33]
8. 0.00189 mol H_2/g VS[a]	0.0265 mol H_2/g VS[a]	Acid (pH 3), 24 h	Anaerobic treatment plant	[34]
9. 0.026.8 mol H_2[a]	0.0341 mol H_2[a]	Acid (pH 3), 30 min	Intertidal zone	[35]
10. 0.00074 mol H_2[a]	0.0071 mol H_2[a]	Acid (pH 3), 24 h	Sewage treatment plant	[22]
Base pretreatment				
11. 1.34 mol H_2/mol glucose	0.38 mol H_2/mol glucose	Base (pH 10), 24 h	Sewage treatment plant	[31]
12. 0.68 mol H_2/mol glucose	0.70 mol H_2/mol glucose	Base (pH 10), 24 h	Sewage treatment plant	[30]
13. 0.10 mol H_2/mol glucose[b]	0.20 mol H_2/mol glucose[b]	Base (pH 12), 30 min	Intertidal zone	[27]
14. 0.00569 mol H_2/g glucose[a]	0.72 mmol H_2/g glucose[a]	Base (pH 10), 24 h	Sewage treatment plant	[47]
15. 0.51 mol H_2/mol hexose	0.3 mol H_2/mol hexose	Base (pH 12), 24 h	POME treatment plant	[21]
16. 0.00037 mol H_2/g COD	0.00012 mol H_2/g COD	Base (pH 12), 24 h	POME treatment plant	[33]
17. 0.00240 mol H_2/g VS[a]	0.0265 mol H_2/g VS[a]	Base (pH 12), 24 h	Anaerobic treatment plant	[34]
18. 0.00006 mol H_2/g VS[a]	0.00005 mol H_2/g VS[a]	Base (pH 12), 5 min	Sewage treatment plant	[45]
19. 0.0154 mol H_2[a]	0.0341 mol H_2[a]	Base (pH 10), 30 min	Intertidal zone	[35]
20. 0.00211 mol H_2[a]	0.0071 mol H_2[a]	Base (pH 11), 24 h	Sewage treatment plant	[22]

[a] H_2 yield was estimated using $PV=nRT$ at standard condition where $P=1$ atm; $R=8.21\times10^{-5}$ m^3 atm/mol K and $T=300$ K.
[b] Estimated value.

Table 5
H_2 production from sludge pretreated with chemical activation and inhibition methods.

Pretreatment condition	H_2 yield	Sludge source	Ref.
Chemical activation			
1. Reactivated in Clostridium enforcement medium, 15 days	2.19 mol H_2/mol hexose	Cattle farm	[17]
2. Loading shock (50 g sucrose/L), 2 days	1.96 mol H_2/mol hexose	POME treatment plant	[21]
3. Loading shock (50 g sucrose/L), 2 days	0.199 mol H_2/L POME	POME treatment plant	[21]
4. Reactivated in rice medium, 1 month	0.00212 mol H_2/g TS[a]	Composting plant	[49]
5. Reactivated in rice medium, 1 month	0.00517 mol H_2/g TS[a]	Composting plant	[49]
6. KNO_3 (10 mmol/L)	0.0345 mol H_2[a]	Intertidal zone	[35]
Chemical inhibition			
7. BES (10 mmol/L), 24 h	0.33 mol H_2/mol glucose	Sewage treatment plant	[31]
8. BES (10 mmol), 30 min	1.01 mol H_2/mol hexose	POME treatment plant	[21]
9. BES (0.2 g/L), 24 h	0.0000317 mol H_2/g COD	H_2 producing reactor	[32]
10. Chloroform (1%), 24 h	0.61 mol H_2/mol glucose	Sewage treatment plant	[31]
11. Chloroform (2%), 24 h	0.00353 mol H_2/g glucose[a]	Sewage treatment plant	[47]
12. Chloroform (0.1%), 24 h	0.00023 mol H_2/g COD	POME treatment plant	[33]
13. Chloroform (0.2%)	0.00134 mol H_2/g VS[a]	Anaerobic treatment plant	[34]

[a] H_2 yield was estimated using $PV=nRT$ at standard condition where $P=1$ atm; $R=8.21\times10^{-5}$ m^3 atm/mol K and $T=300$ K.

2.1.2.2. Chemical activation and inhibition. Chemical activation pretreatment enriches HPB by spiking or shocking the seed sludge with a selected substrate or medium such as sucrose or rice (Table 5, nos. 1–6). This method is useful in terms of enriching selective HPB. For example, seed sludge activated with clostridium enforcement medium can enrich HPB like *Clostridium* spp [17]. It has also been claimed that spiking the seed sludge with sucrose is highly effective in enriching thermophilic HPB such as *Thermoanaerobacterium* sp. [21]. However, this method is practical only if the specific medium or substrate for the targeted HPB is identified which is often challenging.

The chemical inhibition pretreatment can employ toxic chemicals such as chloroform and 2-bromoethanesulfonate (BES) into the seed sludge to inhibit HCB (Table 5, nos. 7–13). However, these inhibitors are often lethal to the HPB [21,31,33,34] and highly toxic and harmful to humans and the environment. Consequently, it is extremely challenging to search for a suitable and yet environmentally benign inhibitor. Therefore, chemical inhibition pretreatment is the least favorable method for enriching HPB.

2.1.3. Combination pretreatments

Combination pretreatment methods combine the strengths of physical and chemical pretreatment methods to improve the selection of HPB. Combination methods employ dual pretreatments such as repeated heating (Table 6, nos. 2 and 6) or a combination of several pretreatments (Table 6). Studies have shown that heat coupled with acid, acid coupled with BES, and heat coupled with ultrasonic treated seed sludge produced at least two fold more H_2 compared to that of individual pretreatments. In addition, the sequence of combination pretreatments plays an important role [50]. For example, it is crucial that heat pretreatment be applied before pretreatment with chloroform [50]. Research has shown that the yield obtained by using heat pretreatment followed by chloroform generated 22% more H_2

Table 6
Hydrogen production from sludge pretreated with combination pretreatment.

Pretreatment	H_2 yield	Sludge source	Ref.
1. Heat (boiling)+aeration (4 min)	1.83 mol H_2/mol glucose	River sludge	[51]
2. Repeated boiling (2× for 5 h)	1.00 mol H_2/mol glucose	Beer Industry	[50]
3. Heat (77 °C)+Ultrasonic (20 min)	1.55 mol H_2/mol glucose	Sewage treatment plant	[30]
4. Heat (repeated boiling)+chloroform (0.05%)	0.51 mol H_2/mol glucose	Beer Industry	[50]
5. Chloroform (0.05%)+heat (repeated boiling)	0.44 mol H_2/mol glucose	Beer Industry	[50]
6. Repeated boiling (2× for 5 h)	0.33 mol H_2/mol glucose	Bakers yeast industry	[50]
7. Heat 90 °C+Ultrasonic	1.32–1.50 mol H_2/g COD[a]	Sewage treatment plant	[40]
8. Acid (pH 5)+BES (0.2 g/L)	2.90×10^{-5} mol H_2/g COD	H_2 producing reactor	[32]
9. Heat (100 °C)+acid (pH 5)	2.07×10^{-5} mol H_2/g COD	H_2 producing reactor	[32]
10. Acid (pH 5)+heat (100 °C)+BES (0.2 g/L)	1.08×10^{-5} mol H_2/g COD	H_2 producing reactor	[32]
11. Heat (100 °C)+BES (0.2 g/L)	8.40×10^{-6} mol H_2/gCOD	H_2 producing reactor	[32]
12. Heat (boiling)+freeze −20 °C+thaw (4 °C)	0.41 mol H_2/mol glycerol	Sewage treatment plant	[52]
13. Heat (95 °C)+acid (pH 3–5), 48 h	0.0545 mol H_2[a]	Cattle farm	[37]
14. Water soak (3 h)+Reactivated in glucose (3 days)	0.011 mol H_2/g substrate[a]	Cattle farm	[53]
15. Aeration (4 days)+Reactivated in glucose (3 days)	0.010 mol H_2/g substrate[a]	Cattle farm	[53]
16. UV (3 h)+Reactivated in glucose (3 days)	0.010 mol H_2/g substrate[a]	Cattle farm	[53]

[a] H_2 yield was estimated using $PV=nRT$ at standard condition where $P=1$ atm; $R=8.21\times10^{-5}$ m^3 atm/mol K and $T=300$ K.

compared to using chloroform followed by heat (Table 6, nos. 4 and 5). This is because HPB sporulates from heat pretreatment. Since spores are more stress-resistant, the subsequent chemical pretreatment further eliminates HCB and enriches HPB. Currently, the best combination pretreatment, which is heat pretreatment followed by aeration pretreatment, has only produced 1.83 mol H_2/mol glucose (Table 6, no. 1). It is interesting to note that the result of this combination pretreatment is still lower than that of heat pretreatment. This method is established as an alternative to physical or chemical methods when individual pretreatments cannot effectively enrich HPB.

2.2. Microbial diversity

Sludge containing diverse microorganisms and functional seed sludge that produces H_2 is usually enriched by pretreatment methods (Supplementary Table S5). Different pretreatment methods have shown different preservation effects on a variety of bacteria [21,29] and this directly influences the H_2 yield. For example, HPB such as *Clostridium acetobutylicum* is predominant in heat pretreated sludge (Supplementary Table S5, nos. 1, 2, 25); *Clostridium* spp. is also found in pH pretreated sludge (Supplementary Table S5, nos. 12, 13 and 23); *Thermoanaerobacterium* sp. is found in load shock pretreated sludge (Supplementary Table S5, no. 9); and *Bacillus* sp. is found in BES pretreated sludge (Supplementary Table S5, no. 11). There are also some HCB such as *Lactobacillus* spp. and *Bifidobacterium* spp. which persist even after pretreatment (Supplementary Table S5, nos. 2, 5, 6, 12). Typically, pretreated seed sludge that contains more varieties of HPB and less of HCB produces a higher amount of H_2 (Table 7, nos. 1–9). For example, seed sludge containing only HPB (Table 7, nos. 1–5) was found to produce a higher amount of H_2 than seed sludge containing both HPB and HCB (Table 7, nos. 6–9). On top of that, seed sludge containing several strains of *Clostridium* spp. also produced a higher amount of H_2 compared to a single strain or pure culture [14,19,26,54]. This is because different bacteria may utilize different substrates or cooperate in breaking down complex substrates in order to produce H_2. This synergistic interaction among a variety of bacteria in seed sludge is more beneficial than a pure culture in terms of H_2 production from complex substrates such as wastewater.

The variety of HPB which belongs to the family of strict anaerobes Clostridiaceae has the greatest potential in H_2 production via dark fermentation [17–19]. Besides high H_2 production, *Clostridium spp.* can also produce H_2 from a wide range of substrates such as maltose, cellobiose, starch, glucose, sucrose, xylose, dextrin, paper cellulose, powder cellulose, casein and ground nut oil [20]. This allows *Clostridium spp.* to produce H_2 from waste streams that contain diverse substrates. *Ethanoligenens harbinens* is a newly identified HPB enriched from aerated seed sludge [22]. It is a strict anaerobe that produces ethanol and H_2 simultaneously. This HPB is highly resistant against the bactericidal effect of ethanol during H_2 production. This suggests that *Ethanoligenens harbinens* can be used in ethanol-rich waste for H_2 production. *Bacillus megaterium* is another newly identified HPB [23] isolated from intertidal sludge and it tolerates high salinity levels of up to 15% [55]. This is useful in H_2 production from high salinity wastewater or even polluted sea water.

In contrast to strict anaerobe, some researchers have suggested that facultative-HPB could be the better H_2 producers. Most of the identified facultative-HPB such as *Enterobacter* spp. and *Klebsiella* spp. [22,24] belong to the family of Enterobacteriaceae. Their higher tolerance to oxygen stress, allows facultative-HPB to act as a shelter for hydrogenase. Hydrogenase can be irreversibly inhibited by oxygen regardless of whether it is present in a strict or facultative-HPB [56,57]. Facultative-HPB is able to recover the activity of hydrogenase by rapidly depleting oxygen which accidentally enters the fermentation medium [57–59]. However, the trade-off to this is that facultative-HPB generates lower amounts of H_2 compared to strict anaerobes such as *Clostridium spp*. Therefore, facultative-HPB in sludge can function as a defense against oxidative stress while maintaining an oxygen free condition for strict anaerobes to produce H_2. This shows that the symbiotic interaction between strict and facultative-HPB in seed sludge is important to sustain H_2 production.

2.3. Effects of operation conditions on hydrogen production by sludge inocula

2.3.1. Effects of substrate

H_2 research aims to integrate dark fermentation with waste management. Therefore, many researchers are focusing on H_2 production from organic waste in various streams of waste (Supplementary Tables S1–S4). Organic substrates found in wastewaters are cheap and easily available. Hence, they can be used in dark fermentation for H_2 production. However, wastewaters are not usually sufficiently nutritious to support H_2 production and it is not practical to continuously supply the fermentation process with costly nutrients such as glucose, peptone and yeast extract. One of the solutions is to improve the nutrient content using a

combination of different types of wastewaters [60]. The production of H_2 significantly increases by combining two different types of waste. For example, food wastewater or cassava stillage is rich in carbohydrate and sewage sludge is rich in nitrogen and other micro-nutrients. When these combined substrates were applied in dark fermentation, H_2 yield increased by 0.63 fold [60]. This shows that mixing carbohydrate and nitrogen-rich substrates improves the nutrient content in fermentations and increases H_2 yield. In addition, waste from different resources contains varieties of bacteria. The synergetic interaction between microflora from different waste resources also contributes to improved H_2 yield from the combined wastes [13]. A combination of wastes from different sources provides an opportunity to enhance H_2 production by improving the nutrient content and microbiological profile in the fermentation system.

A balanced concentration of substrate also plays an important role in H_2 production. It is logical to assume that H_2 production increases with substrate concentration. For example, it was found that when the cellobiose concentration increased 2-fold, the H_2 yield increased from 1.57 to 2.19 mol H_2/mol hexose [17]. A relatively low substrate concentration is only sufficient to support biomass growth and hence H_2 production is restricted [49,61–63]. However, an excessive amount of substrate does not always ensure high H_2 production. This is because an excessive amount of substrate increases osmotic pressure and hence inhibits HPB growth. Furthermore, excess substrate inhibits H_2 production by shifting fermentation pathways to produce alcohol and/or lactic acid. This will be further discussed in the next section [11]. On the other hand, in the case of ineffective sludge pretreatment, a high substrate concentration provokes methane production from methanogens. When the substrate is in excess, it is rapidly converted into H_2 and this leads to the accumulation of H_2. The increase in H_2 partial pressure triggers methane production from methanogens that are still in the sludge because H_2 is the intermediate precursor for methane production [64]. This can be prevented by reducing the substrate input for H_2 production as suggested by Chen et al. [64]. Thus, a reasonable amount of substrate in the fermentation is important because limited or excessive substrates inhibit H_2 production.

Accessibility of HPB to substrates directly influences the sustainability of H_2 production. Simple substrates such as glucose and lactose are easily accessible for H_2 production. Theoretically, 1 mol

Table 7
Type of pretreated sludge that contains only H_2 producing bacteria and both H_2 producing and consuming bacteria.

Source of sludge	Microbial community	Pretreatment	H_2 yield	Ref.
Sludge contains only H_2 producing bacteria				
1. Sewage treatment plant B	*Clostridium acetobutyricum* (AE0011437.1) *Clostridium butyricum* (DQ831124.1) *Clostridium* sp. HPB-21 (AY862509.1) Uncultured *Clostridium* sp. (EF700377.1)	Heat	2.30 mol H_2/mol glucose	[18]
2. POME treatment plant	*Thermoanaerobacterium sp.* (AY999015) *Thermoanaerobacterium thermosaccharolyticum* (AY999014) *Clostridium thermopalmarium* (AF286862)	Loading shock	1.96 mol H_2/mol hexose	[21]
3. Sucrose-based synthetic wastewater sludge	*Clostridium butyricum* CGS5	Heat	2.78 mol H_2/mol sucrose	[19]
4. Sewage treatment plant	*Klebsiella* sp. HE1 (AY540111)	N.A.	0.92 mol H_2/mol sucrose	[24]
5. Sewage treatment plant	*Clostridium acetobutyricum* (FM994940.1) *Klebsiella pneumonia* (GQ214541.1) *Clostridium butyricum* (DQ831124.1) Uncultured bacterium (DQ464539.1 and DQ414811.1)	Heat	0.0106 mol H_2/g carbohydrate	[14]
Sludge contains both H_2 producing and consuming bacteria				
6. POME treatment plant	*Lactobacillus* sp. (AY363384) *Bacillus* sp. (AB193859) *Clostridium* sp. (AB234007)	Acid	0.65 mol H_2/mol hexose	[21]
7. Sewage treatment plant C	*Bacillus* sp. (DQ168845.1) *Clostridium butyricum* (DQ831124.1) *Clostridium acetobutyricum* (DQ235219.1 and FM994940.1) *Clostridium* sp. (DQ168846.1) *Lactobacillus delbrueekii* (FJ915706.1) Uncultured bacterium (DQ235219.1) Uncultured *Bacillus* sp. (DQ168845.1) Uncultured *Clostridium* (DQ168846.1)	Heat	2.18 mol H_2/mol glucose	[26]
8. Sewage treatment plant D	*Bifidobacterium boum* (AY166529.1) *Clostridium* sp. (FJ876436.1) *Clostridium butyricum* (DQ831124.1) *Clostridium acetobutyricum* (FM994940.1) *Lactobacillus fermentum* (GQ131282.1) *Lactobacillus delbrueekii* (FJ915705.1 and FJ915706.1) Uncultured bacterium (AB441617.1)	Heat	1.32 mol H_2/mol glucose	[26]
9. Intertidal sludge	*Bacillus* sp. (GQ180912) *Lactobacillus plantarum* (GQ180905 and GQ180906) *Clostridium* sp. (GQ180907, GQ180908, GQ180910 and GQ180911) *Enterococcus faecium* (GQ180909)	Freeze and thaw	0.15 mol H_2/mol glucose	[27]

of glucose ($C_6H_{12}O_6$) should produce 12 mol of H_2 (Eq. (1)), while 1 mol of lactose ($C_{12}H_{22}O_{11}$) will produce 23 mol of H_2 (Eq. (2)).

$$C_{12}H_{22}O_{11} + 12H_2O \rightarrow \mathbf{23H_2} + 12\ CO_2 \quad (2)$$

Nevertheless, dark fermentation is less efficient in terms of converting substrates to H_2 because most thermal enthalpies are lost in the formation of volatile fatty acids (VFA). Typically, the maximum energy conversion from glucose to H_2 is only 33% via the acetate pathway (Eq. (3)) and 17% via the butyrate pathway (Eq. (4)). Meanwhile the efficiency of lactose conversion to H_2 is only 31% via the acetate and methane pathways (Eqs. (5) and (6)) [65–67].

Glucose fermentation

Acetate pathway: $$C_6H_{12}O_6 + 2H_2O \rightarrow \mathbf{4H_2} + 2CH_3COOH + 2CO_2 \quad (3)$$

Butyrate pathway: $$C_6H_{12}O_6 \rightarrow \mathbf{2H_2} + CH_3CH_2CH_2COOH + 2CO_2 \quad (4)$$

Lactose fermentation

Acetate pathway: $$C_{12}H_{22}O_{11} + 5H_2O \rightarrow \mathbf{8H_2} + 4CO_2 + 4CH_3COOH \quad (5)$$

Methane pathway: $$C_{12}H_{22}O_{11} + 5H_2O \rightarrow \mathbf{8H_2} + 8CO_2 + 4CH_4 \quad (6)$$

However, achieving the theoretical maximum H_2 yield is not feasible. Currently, the highest reported H_2 yield is only 2.3 mol H_2/mol glucose which is only about 50% of the theoretical maximum H_2 yield [18]. This phenomenon is caused by (1) the rapid conversion of substrate into cell biomass instead of H_2 [49,61–63] and (2) an inappropriate combination of fermentation conditions as discussed in this paper. It is postulated that mixed microflora in the seed sludge could overcome this problem via the synergetic interaction among the different bacteria because they can adapt to a wider range of conditions.

2.3.2. Effects of pH

The reported optimum pH for H_2 production is in the range of pH 6–8 (Supplementary Tables S1–S4). This represents the pH range that supports the growth of many HPB including *Clostridium butyricum*, *Clostridium beijerinckii*, *Clostridium tyrobutyricum* and *Clostridium saccharoperbutylacetonicum* [11,19,27,49,68–71]. An optimum pH helps to maintain the surface charge on the cell membrane which facilitates nutrient uptake and hence sustains growth of HPB [11,69]. In addition, HPB contains the essential enzyme, hydrogenase, which plays the most important role in H_2 production. Hydrogenase is reported to function optimally at a pH range of 6–6.5 [72,73]. Evidence of this was seen in a study when H_2 production at a pH level lower than 6 was reduced by half [15] or completely ceased [68]. This shows that pH plays a critical role in sustaining the growth of HPB and the activity of hydrogenase in H_2 production.

It is also noteworthy that the buffer capacity of the fermentation medium plays an important role in regulating the pH in order to achieve optimum H_2 production. Unlike synthetic mediums, natural buffering capacity does not occur in most of the waste resources, hence utilizing waste resources to produce H_2 is hampered [34]. Some researchers have suggested that batch fermentation should be initiated at a higher pH level (pH 8–10) [45,74,75] because high initial pH will buffer acid production accordingly and prevent a sharp pH reduction [69]. Zhao et al. [75] and Lee et al. [76] stressed that the medium will become more acidic over time due to the production and accumulation of organic acids during the fermentation process. Hence, a stable pH in the medium is essential to sustain optimum H_2 production.

2.3.3. Effect of temperature

Temperature determines the physiological activities of HPB. The fermentation temperature for most of the H_2 productions was reported in the mesophilic range (20–45 °C) (Supplementary Tables S1–S4). This is because most of the HPB present in the seed sludge are mesophiles such as *Clostridium* spp., *Enterobacter* spp., and *Bacillus* spp. that grow in this temperature range [19,77,78]. However, H_2 production is only vigorous in a narrow range of temperatures even though HPB may grow in a wide temperature range. For example, Mu et al. [79] detected HPB growth at 33–41 °C but the highest H_2 yield was obtained at 39 °C. From the literature, the most promising temperature range for H_2 production is 35–37 °C [18,29,48]. This suggests that HPB are only physiologically active in a narrow temperature range for H_2 production despite their ability to grow in a wide temperature range.

Furthermore, it is argued that H_2 production at higher temperatures (>45 °C) is favorable. This is because H_2 gas is less soluble at high temperatures [11,80,81]. It is also interesting to note that hydrogenase is reported to function optimally in the range of 50–70 °C despite many HPB being identified as mesophiles [11,80,81]. This leads to the identification of several thermophiles that belong to the *Thermoanaerobacterium* genus which produce H_2 at thermophilic temperatures (>45 °C) [21,37,82]. These bacteria can produce up to 1.96 mol H_2/mol hexose at 60 °C after 48 h [21]. Thus, thermophiles are suitable to be used in warm or even hot wastewater, such as beverage producing, food processing or pulp and paper industries, because they are able to tolerate a high operation temperature. Temperature is a crucial parameter in dark fermentation because temperatures outside the suitable range will restrain H_2 production.

2.3.4. Effects of nutrients and inhibitors

2.3.4.1. Effects of organic acids. Fermentative H_2 production is accompanied by the formation of volatile fatty acids (VFA) such as acetate, butyrate, propionate, lactate, formate and ethanol. Productions of VFA via different fermentation pathways are influenced by the variety of HPB present in the seed sludge which in turn is determined by the pretreatment method. These pathways are indicated by the ratio of acetate to butyrate which is clearly listed in Tables S2–S4 (supplementary data). When the ratio of acetate to butyrate is larger than one, it represents an acetate pathway (Eq. (3)). Meanwhile, a ratio that is smaller than one indicates the butyrate pathway (Eq. (4)). This further emphasizes that H_2 yield is strongly related to the selection of the pretreatment method because this determines the variety of HPB that produces H_2.

In strict anaerobes, the fermentative pathways are divided into two main routes: acidogenesis (acid production) and solventogenesis (solvent production) (Fig. 2). These pathways are usually efficiency indicators of H_2 production [49,83]. Generally, glucose undergoes glycolysis to produce pyruvate with NADH as the electron donor. The electrons generated from the oxidative decarboxylation of pyruvate are transferred to protons and then hydrogenase reduces the protons to molecular H_2 gas. In acidogenesis, the production of acetate is normally the preferred pathway in H_2 production [26,30,49,84]. The ideal H_2 yield is 4 mol/mol of hexose via the acetate pathway but it is halved via the butyrate pathway [49,85–87]. It has been reported that a protein-rich substrate favors the acetate pathway but a carbohydrate-rich substrate favors the butyrate pathway [60]. On the other hand, other acidogenesis pathways which produce VFA such as lactate or propionate have been reported to inhibit H_2 production [26,49,80]. In contrast to acidogenesis, H_2 production in solventogenesis is accompanied by the production of solvents such as ethanol and butanol. However, solventogenesis usually does not encourage high H_2 yield because solvents like ethanol has bactericidal effects [49]. During batch fermentation, the switch from the acidogenesis to the solventogenesis pathway triggers the buildup of biogas partial pressure, the accumulation of

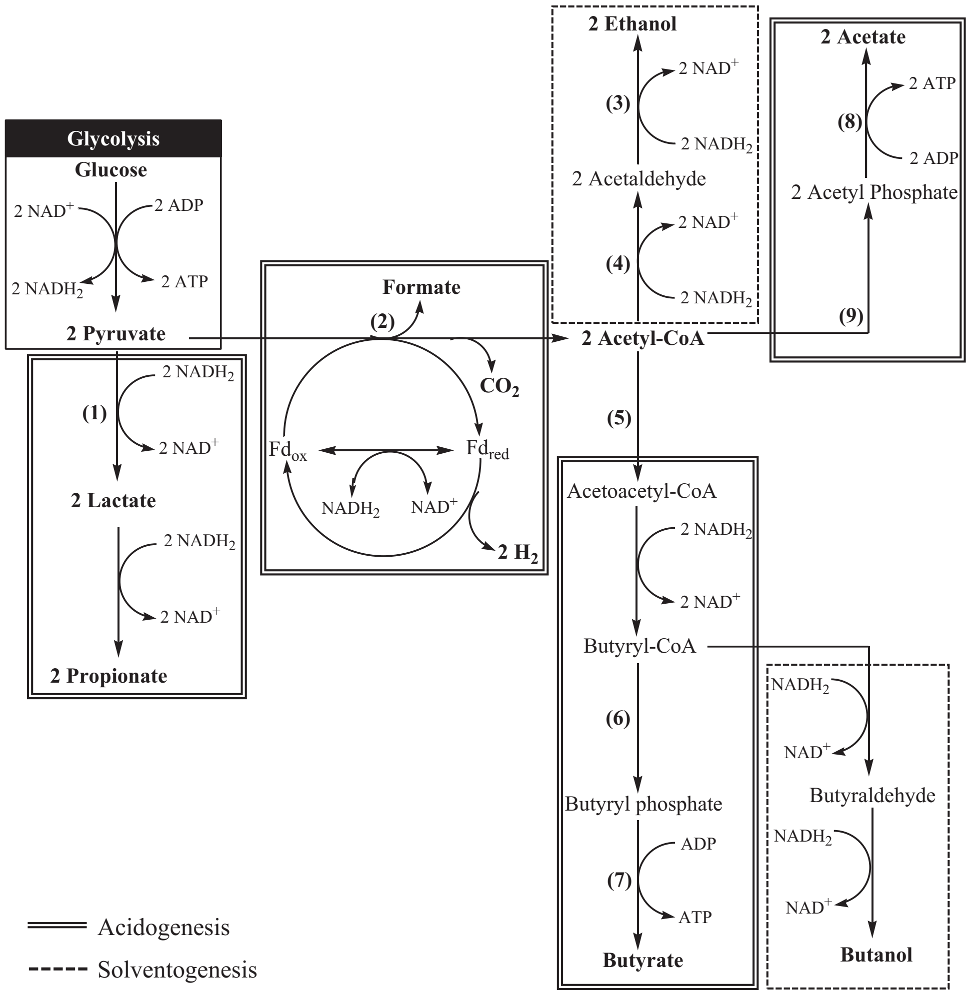


Fig. 2. The connection of glycolytic pathway for glucose fermentation with organic acid and solvent production from pyruvate by strict anaerobes. Numbers in brackets represents key enzymes: (1) lactate dehydrogenase; (2) pyruvate formate lyase; (3) ADH: alcohol dehydrogenase; (4) acetaldehyde dehydrogenase; (5) thiolase; (6) phosphotransbutylase; (7) butyrate kinase; (9) phosphotransacetylase; (8) acetate kinase.

VFA, and the reduction of fermentative pH [8,49,52,88–90]. The direction of the fermentative pathway directly influences the quality of H_2 yield in which the acetate and butyrate pathways are the more favorable directions.

Organic acids can act as a supplementary and/or inhibitory factor to H_2 production. Productions of acetate and butyrate are usually associated with high H_2 production but an accumulation of these acids will negatively impact H_2 production (Fig. 3a). For instance, it has been found that fermentation supplemented with excess butyrate inhibited H_2 production from kitchen waste [84] and acetate inhibited H_2 production from glycerol [52]. Studies have also shown that the H_2 yield from apple pomace was reduced by at least 5% with the addition of acetate and butyrate [41]. In contrast, other organic acids that have been reported as indicators of low H_2 production such as lactate and propionate can be potential supplements when present at a threshold concentration [41]. When added to fermentations, lactate and propionate can trigger a positive reaction to induce higher H_2 production via the pyruvate pathway (Fig. 3b). For example, it was reported that the addition of lactate at a concentration of 650 mg/L enhanced H_2 production by up to 37%; and propionate increased H_2 yield by 28% [41]. The concentration of organic acids in the fermentation medium regulates H_2 production with the control of different feedback mechanisms.

2.3.4.2. Effects of macro- and micronutrients. The macronutrients in dark fermentation are carbon (C), nitrogen (N) and phosphorous (P) and these are usually the essential nutrients [10,16,81,91]. Carbon content is solely contributed by the substrate from which H_2 is produced as discussed in Section 2.2. Nitrogen can be in various forms such as protein, nitrate, nitrite and ammonium. The presence of ammonium in fermentation is essential because it does not only serve as a nutrient for bacterial growth but also provides a slight buffering capacity in the medium against the production of organic acids [92,93]. However, it is argued that nitrogen content is not essential because it does not influence the production of total biogas but might affect the lag time of gas production [52]. Phosphorous is usually present in the form of phosphate. Argun et al. [94] showed that the maximum H_2 was produced at C/P ratio of 1000 (equivalent to C/N/P of 100/0.5/0.1). Consequently, a balanced nutrient level is essential for optimum H_2 production.

Metal ions are micronutrients for fermentation. Nickel (Ni) and iron (Fe) serve as the co-factors for hydrogenase [95]. Hydrogenase is the main enzyme responsible for H_2 production. It is classified according to the metal component in the active site commonly Ni–Fe and Fe–Fe [96,97]. Therefore, fermentation medium supplemented with Ni and Fe enhances H_2 production [13,29,98]. It was reported that a fermentation medium supplemented with 0.1 mg/L Ni resulted in a 2.4 fold increase in H_2 yield compared to

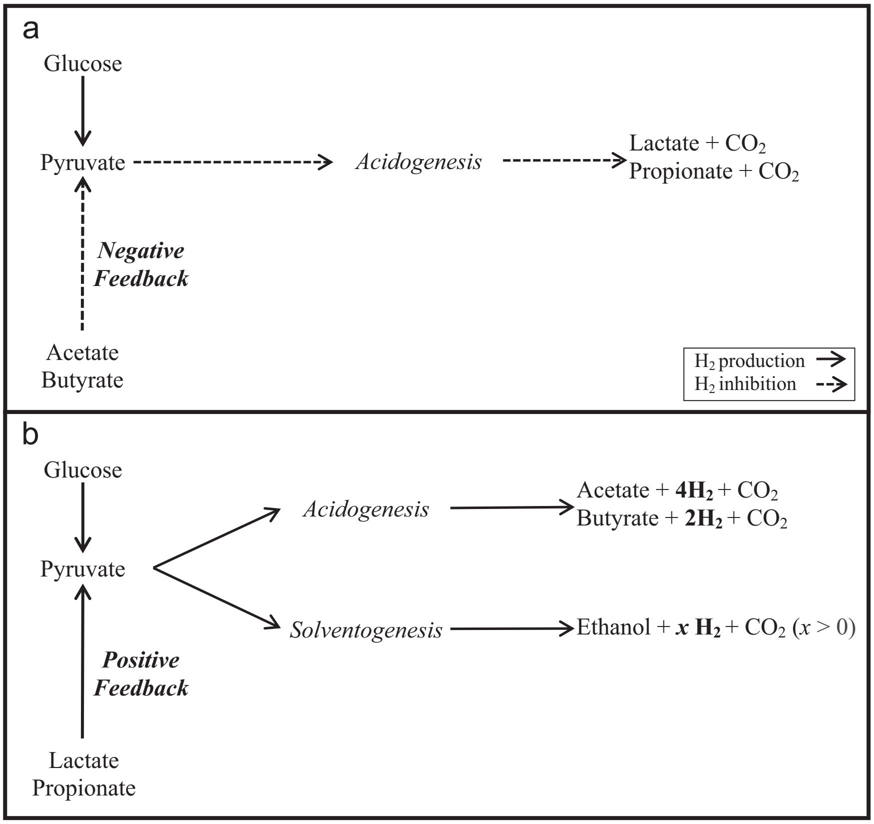


Fig. 3. Relationship between fermentation products and H_2 production. (a) Event of H_2 inhibition due to accumulation of acetate and/or butyrate and (b) event of H_2 production supplemented by lactate and propionate.

Table 8
Summary of factors responsible and recommended conditions for high H_2 production from seed sludge via dark fermentation.

Parameters	Reported range	Recommended conditions
Pretreatment temperature	65–100 °C for 15–90 min	65 °C for 30 min or 100 °C for 15 min
Fermentation pH	pH 6–8	pH 6.0–6.5
Fermentation temperature	20–45 °C	35–37 °C
Micronutrients concentration	N.A.	Ni 0.1 mg/L; Fe 55 mg/L; Zn ≤ 0.24 mg/L; Cu ≤ 3.0 mg/L; Cr ≤ 15 mg/L; Ca 50–150 mg/L; Mo 0.0042 mg/L

non-supplemented fermentations [29], whereas a fermentation medium with increased Fe concentration of 18–55 mg/L improved H_2 yield by 1.5 fold and shortened the lag phase by 0.33 fold [13]. Other metal ions including zinc (Zn), copper (Cu) and chromium (Cr) are also found to be beneficial to other key enzymes including dehydrogenase, dismutase, hydrogenase and methyltransferase [99]. The threshold concentrations of Zn, Cu and Cr are reported as 0.24 mg/L, 3.0 mg/L and 15 mg/L respectively. Once the concentration exceeds the threshold limit, these elements become toxic to HPB. For instance, the yield of H_2 was reduced by half when the concentration of Zn, Cu and Cr exceeded the threshold concentration [100]. Additional metal ions at appropriate concentrations can enhance H_2 production accordingly by regulating the activity of the enzymes involved in the process.

Metal ions can also stabilize H_2 production and improve the H_2 production processes [15,95]. Calcium (Ca) concentration in the range of 50–150 mg/L stabilizes and improves H_2 production [13,101,102]. Adding molybdate (Mo) favors the H_2 production process because it inhibits sulfate reduction and methane production [98,103]. Niu et al. [15] reported that a low concentration of Mo (0.0042 mg/L) could increase H_2 yield by 29%. Overall, metal ions assist in obtaining high H_2 yield by alleviating fermentation conditions.

3. Conclusion

The benchmark of H_2 production from sludge via dark fermentation is summarized in Table 8. The selection of pretreatment methods determines the variety of HPB preserved in the seed sludge. The activity of HPB is influenced by various fermentation conditions, including type of substrate, fermentation pH and temperature, and types of nutrients, supplements and inhibitors. With the identification of the strengths and weaknesses of these conditions, we can further enhance H_2 production via dark fermentation.

Hydrogen production from seed sludge via dark fermentation can be a sustainable approach for long term fuel supply. We have presented the importance of sludge enrichment using different pretreatment methods and have revealed that heat pretreatment is the most frequently applied and the most effective method to eliminate HCB while preserving HPB. In addition, the enriched sludge requires optimum fermentation conditions in order to produce H_2 optimally through the correct fermentation pathway.

However, the current fermentation conditions only enable the enriched sludge to produce up to 2.3 mol H_2/mol glucose via dark fermentation. This is still far from the theoretical value of 4 mol H_2/mol glucose. To further enhance the H_2 yield from seed sludge as inoculum, the challenges ahead are to investigate

(1) The type of pretreatment methods with appropriate condition and duration that can effectively enrich HPB in seed sludge in order to achieve maximum H_2 production;
(2) The combination of fermentation conditions that can direct HPB into the correct fermentation pathway for optimum H_2 production.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.rser.2014.03.008.

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